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A critical domain of the *Cucumber mosaic virus* 2b protein for RNA silencing suppressor activity

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ABSTRACT

Alignment of *Cucumber mosaic virus* (CMV) 2b protein sequences from two CMV subgroups revealed two highly variable regions. To examine contributions of variable sequence domains to the suppressor activity, we performed a comparative study between 2b proteins of a subgroup I strain (SD-CMV) and a subgroup II strain (Q-CMV). Here we show that the suppressor activity of SD2b is stronger than that of Q2b and that a domain existent in SD2b but absent in Q2b is a major determinant of the suppressor activity of SD2b. We further show that the same domain is responsible for inhibition of *Nicotiana benthamiana* AGO4-1 transcription. Our results implicate AGO4 as a mediator for CMV 2b to suppress systemic silencing and DNA methylation.

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1. Introduction

RNA silencing is one of the natural plant defense mechanisms against virus infection [1,2]. A current model for antiviral silencing in higher plants, taking *Arabidopsis thaliana* as an example, suggests that double-stranded (ds) RNA replication intermediates of viral genomic RNAs or highly structured regions within single-stranded viral RNAs are first cleaved by RNase III-type Dicer-like 4 (DCL4) or alternatively by DCL2 to produce 21- or 22-nucleotide (nt) small interfering RNAs (siRNAs). One strand (the guide strand) of the viral siRNA duplexes is then incorporated into the RNA-induced silencing complex (RISC) which contains an Argonaute protein (AGO), notably AGO1, as a core component, and ushers the activated RISC to target viral RNAs via sequence complementarity and degrade viral RNAs by the slicing activity of AGO1 [3]. As a counter-defense strategy, plant viruses produce a variety of suppressor proteins to inhibit the host antiviral silencing at almost every key step in the RNA silencing pathway, including production and processing of viral dsRNAs, stabilization and amplification of viral siRNAs, assembly of RISC, and slicing of target viral RNAs [4]. Several viral suppressors can also interfere with microRNA (miRNA)-mediated host mRNA cleavage to disrupt normal plant development [3,4] [and references therein].

Cucumber mosaic virus (CMV) 2b, encoded by a 3'-proximal open reading frame of RNA 2 of the tripartite viral RNA genome [5], was one of the first identified RNA silencing suppressors [6] and has attracted extensive research in the past decade on multiple aspects of its suppressor functions and the mechanisms underlying these activities. CMV 2b has been shown to inhibit siRNA-directed systemic silencing [7], and also to affect local silencing [8,9]. These suppressor activities might be related to its ability to bind siRNAs [7,9]. CMV 2b may also interfere with siRNA-mediated translational repression [8].

More recently, it has been shown that the 2b protein from a severe CMV strain can perturb miRNA-mediated cellular RNA turnover [10,11] by directly interacting with the AGO1 protein and blocking AGO1's slicer activity [10]. Additionally, CMV 2b can interfere with salicylic acid-mediated virus resistance, likely by reducing accumulation of antiviral silencing-associated viral siRNAs produced largely by the host RNA-dependent RNA polymerase 1 which is salicylic acid-inducible [12–14]. Interestingly, CMV 2b can also significantly reduce transgene de novo DNA methylation, inferring its potential role in interfering with transcriptional gene silencing [7].

The structural bases of RNA silencing suppressor activities have been characterized for several viral suppressors, including three plant viral suppressors, P19 of tombusviruses, P21 of a closterovirus and 2b of *Tomato aspermy cucumovirus* [15–18]. However, the structural motifs responsible for the suppressor activities of CMV 2b are largely unknown, except for the nuclear localization signal (NLS) [19] and a single amino acid (aa) resided outside of the

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NLS(s) but still within the arginine-rich region (ARR) located in the N-terminal half of the 2b protein [9]. Based on RNA sequence data, CMV strains can be classified into three subgroups, IA, IB and II [20]. It has been found that subgroup I strains are, in general, more virulent than subgroup II strains and some studies have suggested that this differential virulence is mediated by the 2b gene [21]. Alignment of aa sequences of 2b proteins from 39 CMV strains revealed that 2b proteins of subgroup I (A plus B) strains are typically longer (110–111 aa) than those of subgroup II (100 aa) [5,22] (Supplementary Fig. S1). These two size-classes of 2b sequences share two highly conserved regions (the ARR harboring NLSs [23] and the C-terminal region) and two less conserved regions flanking the ARR, but differ significantly in the remaining two parts of the protein, i.e. the N-terminal end and a long middle portion before the highly conserved C-terminus. The latter highly variable region contains gaps of about 10 aa in the 2b proteins of subgroup II according to the sequence alignment. These subgroup-distinctive sequence features provide us with a good platform to study the contribution of the variable sequence domains to the differential suppressor activities of CMV 2b. Here we report a comparative study of the 2b proteins from a severe subgroup IB strain Shandong (SD-CMV) and a mild subgroup II strain Q (Q-CMV). We show that the suppressor activity of SD2b is stronger than that of Q2b and that a variable domain of SD2b, which is absent in Q2b, is a major contributor to the stronger activity of SD2b. We further show that the same domain is also responsible for the stronger activity of SD2b in suppression of expression of the tobacco *AGO4-like* gene, consistent to the roles of 2b in inhibiting systemic silencing and RNA-directed DNA methylation (RdDM).

2. Materials and methods

2.1. Protein sequence analysis

The 2b sequences were aligned using ClustalW 1.82. Protein secondary structure prediction was performed by using PSIPred (<http://bioinf.cs.ucl.ac.uk/psipred/>) and NNPREPREDICT (<http://www.cmpharm.ucsf.edu/cgi-bin/nnpredict>).

2.2. Plasmids construction

Plant binary vectors for agroinfiltration were derived from the pCambia 1300. p35S-SD2b and p35S-Q2b have been described in [24]. The chimeric QSD1-encoding sequence was PCR-amplified from p35S-Q2b by a forward primer 5'-CGGGATCCATGGAATTGACGAAGGCGCAGTGTGACCGCCGACCTCC-3' which contained a stretch of nucleotides coding for the N-terminal 8 aa of SD2b (italicized) and a reverse primer: 5'-GCGAGCTCTCAAAACGACCTTCGGCCCAT-3', and inserted into p35S-Q2b to replace the Q2b sequence to generate p35S-QSD1. To obtain chimeras QSD3 and QSDA3, the N-terminal Q2b (aa 1–61)-encoding sequence was amplified from p35S-Q2b with a forward primer 5'-GCTCTAGACCATGGATGTGTTGACAGTAGTGG-3' and a reverse primer 5'-CGGGATCCATCTACTCCGTGGAATGG-3'. Sequences coding for the C-terminal SD2b (aa 64–111 for QSD3 and aa 72–111 for QSDA3) were amplified from p35S-SD2b with a common reverse primer 5'-GCGGAGCTCTCAGAAACGACCTTCGCCCCACTCG-3' and a forward primer 5'-CGGGATCCGAAGTATAGAGATG-3' (for QSD3) or another forward primer 5'-CGGGATCCGTGAACATGGTGGGATTGTCCG-3' (for QSDA3). The DNA fragments coding for the N- and C-portions were fused via a BamHI site corresponding to codons for aa 62 and 63 (GS) of SD2b. For the deletion mutant SDΔ3 in which aa 64–71 was deleted from SD2b, an amplified DNA fragment coding for aa 1–63 of SD2b (including a BamHI site) was linked to the fragment coding for SD2b aa 72–111 (the same

fragment used for construction of QSDA3) through the BamHI site. For construction of SDQ2 in which aa 48–61 of SD2b was replaced by the corresponding motif of Q2b, a specific reverse primer 5'-CGGGATCCATCTACTCCGTGGAATGGAACATCTGGAAAAGTCTCGCTATAGATCGCGCTCTCTC-3' which introduced codons for Q2b aa 48–61 (italicized) was used to amplify the sequence for the N-terminal part of SD2b, which was then linked to the DNA fragment coding for SD2b aa 64–111 via the BamHI site.

The plant expression vector with a C-terminal triple HA tag, pRT104.C-3HA, kindly given by Dr. Markus Fauth (Universität Frankfurt, Department of Molecular Cell Biology, Frankfurt, Germany), was used to clone PCR-amplified products of SD2b and Q2b in a NcoI site. The expression cassettes were then moved into pCambia1300 to generate p35S-SD2b-3HA and p35S-Q2b-3HA. pCambia1300 derivatives expressing HA-tagged chimeric 2b proteins were constructed in a similar way.

The full-length cDNA clone of PVX, pP2C2S [25], kindly provided by Dr. David Baulcombe (The Sainsbury Laboratory, John Innes Centre, Norwich, UK), was used to construct recombinant PVX-2b clones. PCR-amplified sequences coding for SD2b, Q2b and their chimeras or deletion derivatives were separately cloned into p35S-P2C2S [26]. All the 35S promoter-driven recombinant PVX-2b viral vectors were transformed into *Agrobacterium* EHA105 for agroinoculation.

2.3. CMV strains

SD-CMV was isolated from tobacco in Shandong Province of China and propagated in *Nicotiana tabacum* Samsun. Q-CMV was kindly provided by Dr. Huishan Guo and propagated in *N. glutinosa*.

2.4. *Agrobacterium* infiltration

N. benthamiana GFP transgenic line 16c was kindly provided by Dr. David Baulcombe. *Agrobacterium*-mediated transient expression in *N. benthamiana* or *N. tabacum* leaves was conducted by pressure infiltration essentially as described in [27,28]. For co-infiltrations, the *Agrobacterium* culture of the GFP-expressing strain was adjusted to OD₆₀₀ of 0.8 and those expressing various forms of 2b to OD₆₀₀ of 1.2.

2.5. RNA isolation, RT-PCR and quantitative real-time PCR analysis

Fresh leaf tissues (100–200 mg) was ground in liquid N₂ and extracted with Trizol reagent (Invitrogen). RNA concentration was measured by Nanodrop (Thermo, USA). DNase treatment, reverse transcription (RT) reaction and real-time PCR were performed as described [24]. All samples were run in triplicates. Primers 5'-AGTGGAGAGGGTGAAGGTGATG-3' (forward) and 5'-TGATCTGGGTATCTTGAAGAGC-3' (reverse) were used for GFP mRNA analysis, and primers 5'-AGTGGCCGTCAATATCTC-3' (forward) and 5'-TAATGTGCTCAGGCTTCC-3' (reverse) were used for analysis of the *N. benthamiana* AGO4-1 mRNA (GenBank accession number DQ321490). The *N. benthamiana* EF1α mRNA served as an internal control as described [24].

2.6. GFP imaging and Western blot analysis

GFP imaging is described in [24]. To prepare protein samples for Western blot analysis, a leaf patch (50 mg) was ground in liquid N₂ and extracted with 200 μl of 8 M urea. SDS-PAGE and immunoreactions with antibodies against GFP, CMV CP and the HA tag were described in [24]. The monoclonal antibody against HA was bought from TIANGEN (Beijing, China).

3. Results and discussion

3.1. SD-CMV is more efficient than Q-CMV in inhibiting systemic silencing

Suppression of long distance spread of the antiviral RNA silencing represents a viral counter-defensive ability to establish virus infection far beyond the initial invasion sites. To see if a severer CMV strain has a stronger activity against systemic silencing, we compared SD-CMV and Q-CMV in their ability to reverse silenced GFP expression in systemic leaves of *gfp*-transgenic *N. benthamiana* 16c plants which had been infiltrated with an *Agrobacterium* strain harboring p35S-GFP (35S:GFP) to completely silence the GFP expression in the plants (Fig. 1A, a and b). The GFP-silenced 16c plants were inoculated with SD-CMV or Q-CMV of the same concentration (50 µg/ml). At 10 dpi, the newly emerging leaves of SD-CMV-infected plants displayed bright GFP fluorescence (Fig. 1A, c), while those of Q-CMV-infected plants showed only weak GFP fluorescence (Fig. 1A, d). Western blot analysis of the GFP protein level confirmed the differential reversal of GFP expression in systemic leaves by SD-CMV or Q-CMV infection (Fig. 1B, top panel). These results indicated that SD-CMV was more efficient than Q-CMV to suppress systemic RNA silencing in *N. benthamiana*

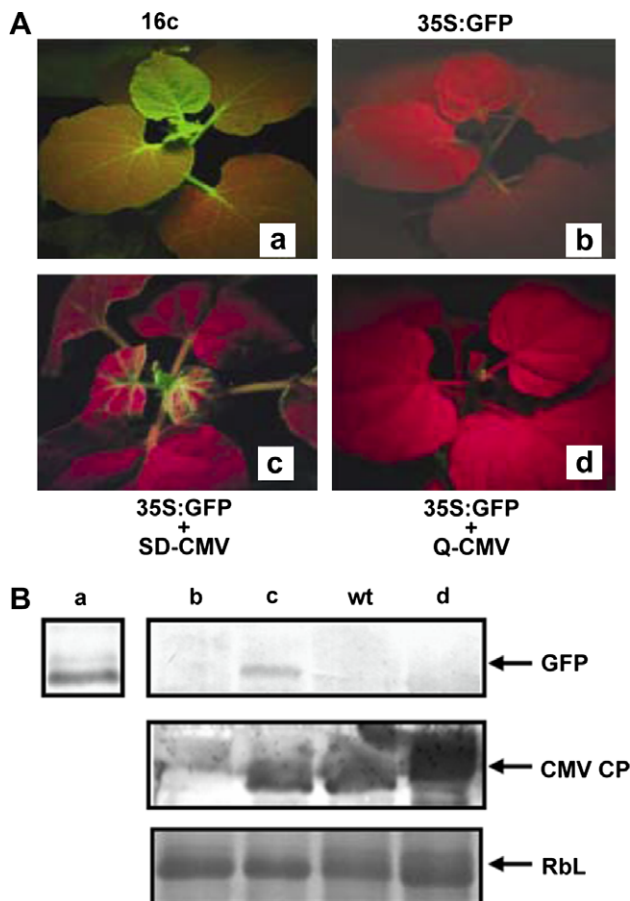


Fig. 1. Differential inhibition of systemic silencing of GFP expression by infection with SD-CMV or Q-CMV. (A) GFP fluorescence in *gfp*-transgenic *N. benthamiana* 16c plants. (a) Untreated 16c plants. (b) 16c plants infiltrated with 35S:GFP. (c and d) GFP-silenced 16c plants infected with SD-CMV (c) or Q-CMV (d) at 10 dpi. (B) Western blot analyses of the GFP protein and CMV CP. Total protein was extracted from leaves of plants shown in A (systemic leaves of plants in c and d), and from systemic leaves of SD-CMV-infected WT *N. benthamiana* plants (wt), and the protein samples were immuno-probed with anti-GFP antibodies (top panel) or anti-SD-CMV CP antibodies (middle panel). Coomassie blue staining of the large subunit of RUBISCO (RbL) served as a protein loading control (bottom panel).

plants and provided direct evidence that the differential virulence of CMV strains is correlated with their ability to inhibit long range silencing signaling, an important viral function that has been attributed to CMV 2b [7]. We also observed that similar levels of SD-CMV coat protein (CP) and Q-CMV CP were accumulated in systemic leaves of respective plants (Fig. 1B, middle panel), further suggesting that the activity of CMV on suppressing systemic RNA silencing is not associated with the ability of the virus to spread to and replicate in systemic leaves.

3.2. SD2b displayed stronger activity than Q2b in suppression of local silencing

Having shown that SD-CMV is more efficient than Q-CMV in inhibiting systemic GFP silencing and this differential suppressor activity might be determined by their encoded 2b proteins, we next sought to compare the activity of SD2b and Q2b in suppressing local RNA silencing. The *Agrobacterium*-mediated transient

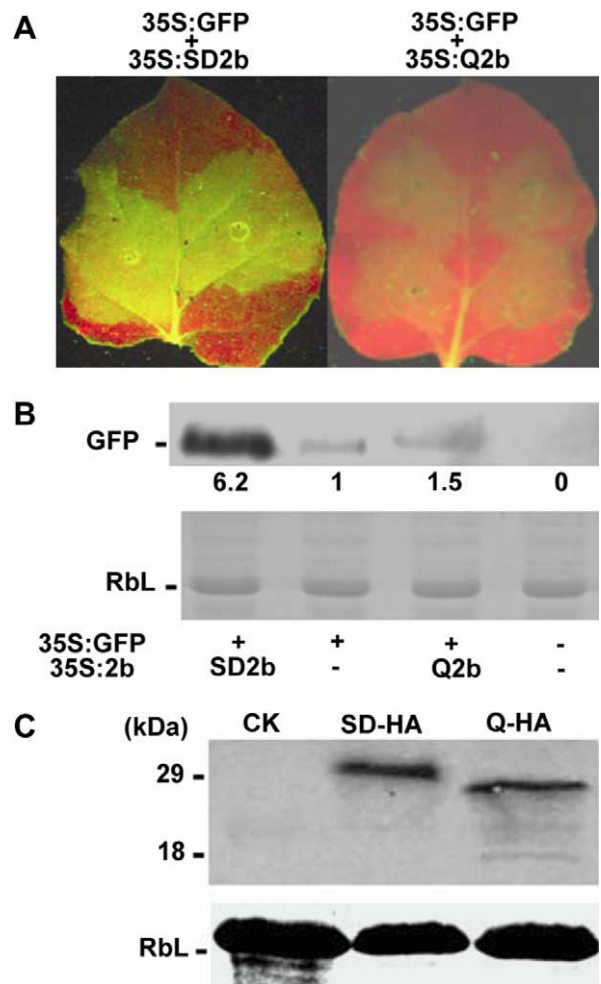
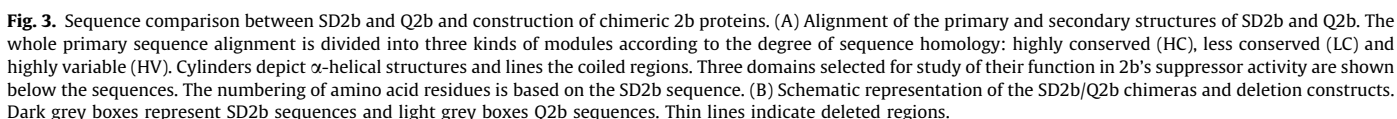


Fig. 2. SD2b is more efficient than Q2b in suppression of local silencing. (A) *Agrobacterium*-mediated transient GFP expression assay. *N. benthamiana* 16c leaves were co-infiltrated with 35S:GFP plus 35S:SD2b or 35S:Q2b. GFP fluorescence was observed at 4 days after co-infiltration. (B) Western blot analysis of the GFP protein in *N. tabacum* SR1 leaves co-infiltrated with 35S:GFP plus 35S:SD2b or 35S:Q2b. Protein samples extracted from leaves infiltrated with 35S:GFP alone or buffer were used as controls. The numbers below the GFP panel refer to the GFP protein levels relative to the 35S:GFP control. Coomassie blue staining of RbL indicates equal loading of each lane. (C) SD2b and Q2b accumulated at similar levels in WT *N. benthamiana* leaves agroinfiltrated with p35S-SD2b-3HA (SD-HA) or p35S-Q2b-3HA (Q-HA). Leaf protein samples were subjected to Western blot analysis probed with the anti-HA monoclonal antibody. CK, total protein from mock-infiltrated leaves. Coomassie blue-stained RbL is shown as a loading control.



3.3. Domain 3 of SD2b is a major contributor to the higher suppressor activity of SD2b

lack of specific domains (Fig. 3B) and analyzed their suppressor activities by the *Agrobacterium*-mediated transient assay performed on WT *N. benthamiana* leaves co-infiltrated with 35S:GFP as described in the last section. Suppressor activities were evaluated by the intensity of the visual GFP fluorescence (Fig. 4A), and more quantitatively, by the accumulation level of the GFP RNA in infiltrated leaves determined by RT-PCR (Fig. 4B). It should be noted that all the examined 2b variants were expressed at similar levels in agroinfiltrated WT *N. benthamiana* leaves shown by a Western blot analysis on HA-tagged proteins although some of them that lack the SD2b domain 3 were partially degraded (Fig. 4C).

QSD1, in which domain 1 of Q2b was replaced with SD2b domain 1, produced moderate GFP fluorescence slightly stronger than Q2b but significantly weaker than SD2b did. Consistently, the GFP RNA level induced by QSD1 was 6.5-fold higher than that induced by Q2b and 9.1-fold lower than that enhanced by SD2b, indicating domain 1 of SD2b contributes to some degree to the higher suppressor activity of SD2b. A similar role could be assigned to domain 2, since introduction of Q2b domain 2 into SD2b at the original place of SD2b domain 2 (resulting in SDQ2) only slightly reduced the GFP fluorescence and retained 44% of the amount of the GFP RNA when compared with SD2b. More dramatic changes in suppressor activity were seen in the recombinant 2b proteins where domain 3 was involved. Fusion of the SD2b segment of aa 62–111 immediately downstream to the Q2b sequence of aa 1–61 created the chimera QSD3 which contained domains 1 and 2 from Q2b and HV2 (including domain 3) and the conserved C-terminal region from SD2b. QSD3 exhibited significantly higher suppressor activity than that of Q2b as it generated bright GFP fluorescence and an 8.8-fold increase in the GFP RNA level, indicating that SD2b domain 3 plays a major role in conferring the stronger suppressor activity of SD2b. This conclusion was reinforced by the data of the following loss-of-function experiments. Deletion of domain 3 from QSD3, resulting in QSDΔ3, almost completely destroyed the gained suppressor activity of QSD3 over Q2b, despite the presence of the C-terminal aa 72–111 sequence of SD2b. Similarly, removal of domain 3 from SD2b, which formed SDΔ3, caused a great decrease in GFP fluorescence and a 95% reduction in the accumulation level of the GFP RNA.

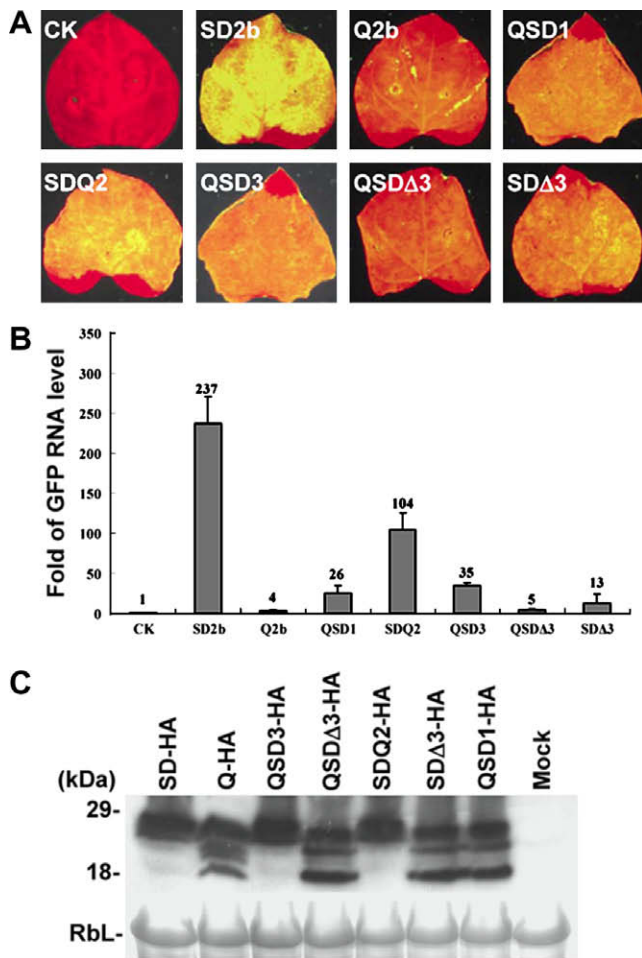


Fig. 4. Suppression activity of chimeric 2b proteins and deletion derivatives. (A) *Agrobacterium*-mediated transient GFP expression assay performed on WT *N. benthamiana* leaves as described in Fig. 2A. CK, leaves infiltrated with 35S:GFP alone. GFP fluorescence was observed 5 days after infiltration. (B) GFP RNA levels in agroinfiltrated leaves described in A. GFP RNA levels were determined by real-time RT-PCR. The level of the *N. benthamiana* *EFL* transcript was used as a normalization control. Error bars represent S.D. of three independent determinations. (C) Expression of HA-tagged 2b variants in agroinfiltrated WT *N. benthamiana* leaves. Western blot analysis was performed as described in Fig. 2C.

The important function of domain 3 in SD2b suppressor activity might be implicated by its unique feature in the high-order structure of the protein. The predicted secondary structure of the SD2b protein indicated that the seven amino acids (63–69) of domain 3 formed an additional short α -helix compared to that of Q2b (Fig. 3A). This third short α -helix might help stabilize the SD2b protein (Fig. 4C) or a complex structure formed by the first two α -helices to bind siRNA or its dsRNA precursor, like α 3 of the flock house virus B2 suppressor did [29,30], or enhance the interaction of 2b with other RNA silencing partner protein like AGO1 to exert the suppressor activity [10].

Taken together, the above data indicate that the three examined variable domains contribute to the difference in suppressor activity between SD2b and Q2b differentially and SD2b domain 3 which is absent in Q2b is a key determinant to account for higher suppressor activity of SD2b.

3.4. CMV 2b inhibits AGO4 transcription and SD2b domain 3 is crucial for the downregulation

RdDM is a transcriptional gene silencing pathway that contributes to transposon silencing and regulation of genes important for

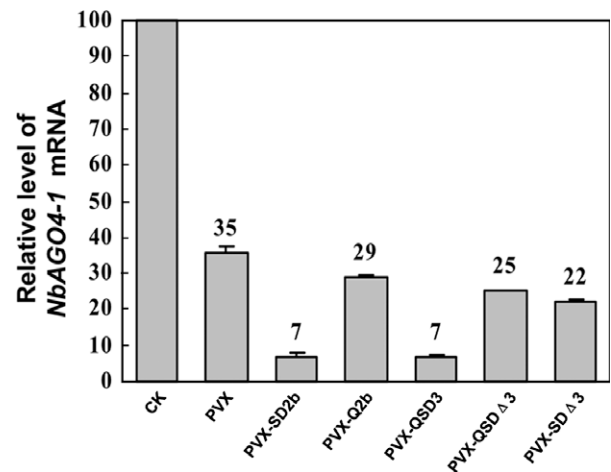


Fig. 5. Inhibition of AGO4 expression by CMV 2b. The levels of the *NbAGO4-1* transcript in *N. benthamiana* leaves agroinfected with recombinant PVX expressing SD2b, Q2b, QSD3, QSDΔ3 or SDA3, or the vector PVX, were determined by RT-PCR and normalized against the level of the *N. benthamiana* *EF1α* transcript. The level of the *NbAGO4-1* transcript in mock-infected leaves (CK) was set as 100%. Error bars represent S.D. of three independent determinations.

plant development and stress responses [31]. AGO4 is a prime component of the RdDM machinery [32,33]. Earlier studies have suggested that CMV 2b (Q2b) is a potent inhibitor of RdDM [7]. To gain some insight into the mechanism of this inhibition, we analyzed the influence of CMV 2b on the expression of the *N. benthamiana* AGO4-like gene (*NbAGO4-1*) [34]. For this purpose, the coding sequences of SD2b and Q2b were separately cloned into the PVX-based plant binary expression vector (p35S-P2C2S) [25,26] to form pPVX-SD2b and pPVX-Q2b. To examine the effect of SD2b domain 3 on the *NbAGO4-1* expression, PVX vectors expressing QSD3, QSDΔ3 and SDA3, named pPVX-QSD3, pPVX-QSDΔ3 and pPVX-SDA3, respectively, were also constructed. All the PVX-2b recombinant vectors, as well as p35S-P2C2S which, albeit without the CMV 2b sequence, harbors a viable gene coding for the silencing suppressor p25 of PVX, were used to infect WT *N. benthamiana* leaves through agroinfiltration. At 11 dpi, the level of the *NbAGO4-1* transcript in *N. benthamiana* leaves infected with each PVX derivative was determined by real-time RT-PCR and compared with that in mock-infiltrated leaves. The results are depicted in Fig. 5. Infection with the vector PVX caused a 65% decrease in the level of the *NbAGO4-1* transcript and infection with PVX-2b recombinants resulted in a further reduction of 28% in the case of PVX-SD2b or 6% with PVX-Q2b, indicating that CMV 2b can inhibit the transcription of *NbAGO4-1* and that the inhibitory effect is correlated to its suppressor activity. This conclusion was supported by the data obtained from similar experiments performed on *N. tabacum* SR1 plants: the level of *NtAGO4-1* mRNA decreased to 15% and 45% in SD-CMV- and Q-CMV-infected plants respectively, when compared to that in mock-inoculated plants (data not shown). Moreover, the data from infection of recombinant PVX expressing the chimeric QSD3 or the deletion variants QSDΔ3 and SDA3 clearly illustrated a critical importance of SD2b domain 3 to the inhibition of *NbAGO4-1* transcription, similar to the role it played in suppression of post-transcriptional gene silencing shown in the last section. We thus furnish a line of evidence that CMV 2b inhibits RdDM by downregulation of AGO4 expression and suggest that the suppression of AGO4 by CMV 2b and PVX p25 probably also mediate their inhibition of systemic silencing [7,35], which was shown to require AGO4 as one of the components in the pathway [34,36]. In this context, we speculate AGO4 may serve as a converging point for cross talk between the transcriptional and post-transcriptional silencing pathways.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.febslet.2008.11.031](https://doi.org/10.1016/j.febslet.2008.11.031).

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